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14. ABSTRACT

Estrogen receptor alpha (ER) is an epigenetically regulated gene. Inhibitors of DNA methyltransferases (DNMT) and histone deacetylases (HDAC) synergistically activate the methylated ER gene promoter in ER negative MDA-MB-231 human breast cancer cells. Using chromatin immunoprecipitation (ChIP), we examined the chromatin status and repressor complex associated with silenced ER and changes in the key regulatory factors during reactivation by inhibitors of DNMT (5-aza-2'deoxycytidine or 5-aza-dC) and HDAC (trichostatin A or TSA). The silencing of ER due to CpG hypermethylation correlates with binding of specific methyl-binding proteins, DNA methyltransferases and HDAC proteins. Inhibition of HDAC activity by TSA results in the increased accumulation of hyperacetylated core histones. The activation of ER gene expression by 5aza-dC also involves the release of the repressor complex involving various methyl binding proteins, DNMTs, and HDAC1. HDAC and DNMT inhibitors also modulated histone methylation at H3-K9 and H3-K4 to form a more open chromatin structure. Together our results impart a better understanding of molecular mechanisms of chromatin remodeling during ER reactivation by DNMT and HDAC inhibitors.

15. SUBJECT TERMS
Estrogen receptor, breast cancer, methyl-CpG binding proteins, histone deacetylase

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INTRODUCTION

Normal mammary development as well as the initiation and progression of breast cancer are intensely influenced by hormonal factors. Breast tumors expressing estrogen receptor alpha (ER) respond well to therapeutic strategies directed at the ER or its ligands whereas those that lack ER do not (1,2). It is imperative to understand what factors determine ER expression in breast cancer. Earlier studies have shown that the ER promoter is hypermethylated and ER mRNA is absent in some ER-negative breast cancer cells. Treatment of ER-negative breast cancer cells with DNA methyltransferase (DNMT) and/or histone deacetylase (HDAC) inhibitors leads to the reactivation of expression of ER mRNA and functional protein, underscoring the importance of DNMTs and HDACs in maintaining the repressive environment at target genes like ER (3,4). The mechanisms involved in suppression of transcription of genes via hypermethylation at CpG islands and histone modifications are an area of active research (5). CpG island hypermethylation may inhibit transcription by interfering with the recruitment and function of basal transcription factors or transcriptional coactivators. Also, hypermethylation of CpG dinucleotides near the transcriptional regulatory region may initiate the recruitment of the methyl-CpG binding domain (MBD) family proteins that mediate silencing of genes via facilitation of a repressive chromatin environment (6,7). At least five methyl-CpG binding proteins including MeCP2, MBD1, MBD2, MBD3 and MBD4 have been identified in vertebrates (8-13). While MeCP2, MBD1, MBD2 and MBD3 can all recruit HDAC-containing repressor complex, distinctive features of each of these proteins have been reported (10, 14-23). Gel shift assays showed that MBD1, MBD2 and MBD4 bind specifically to a variety of DNA sequences containing methyl CpG whereas MBD3 does not either in vitro or in vivo (19-21). Several DNMTs that initiate methylation at position 5 of cytosines of CpG dinucleotides have been identified (24). DNMT1, the chief enzyme responsible for maintenance of mammalian DNA methylation during DNA replication using hemimethylated DNA, can also bind HDAC2 and DMAP1 (DNMT associated protein) to mediate transcriptional repression (25). The de novo methylases, DNMT3a and DNMT3b (that are encoded by different genes (26, 27)), can act as transcriptional repressors by using their ATRX domain to recruit HDAC1 (28, 29). Heterochromatic structure characterized by differential modifications of histones is another facet of the complex machinery influencing repression. Amino terminal tails of the core histones undergo modifications such as acetylation at lysine, methylation at lysine or arginine and phosphorylation at serine to evolve a histone code

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for transcriptional activation and repression (30, 31). These posttranslational modifications modulate the chromatin structure by altering the electrostatic interactions between histone proteins and DNA and also by modifying the recruitment of various non-histone proteins such as coactivators and corepressors to chromatin. The findings that CpG methylation of the ER promoter results in transcriptional silencing (32) and inhibition of HDAC and/or DNMT activity reactivates ER (3,4) support a model in which methyl-CpG binding proteins, DNMTs, and HDACs might be involved in transcriptional control of ER. Chromatin immunoprecipitation (ChIP) was used to monitor the epigenetic determinants of ER chromatin remodeling associated with ER expression.
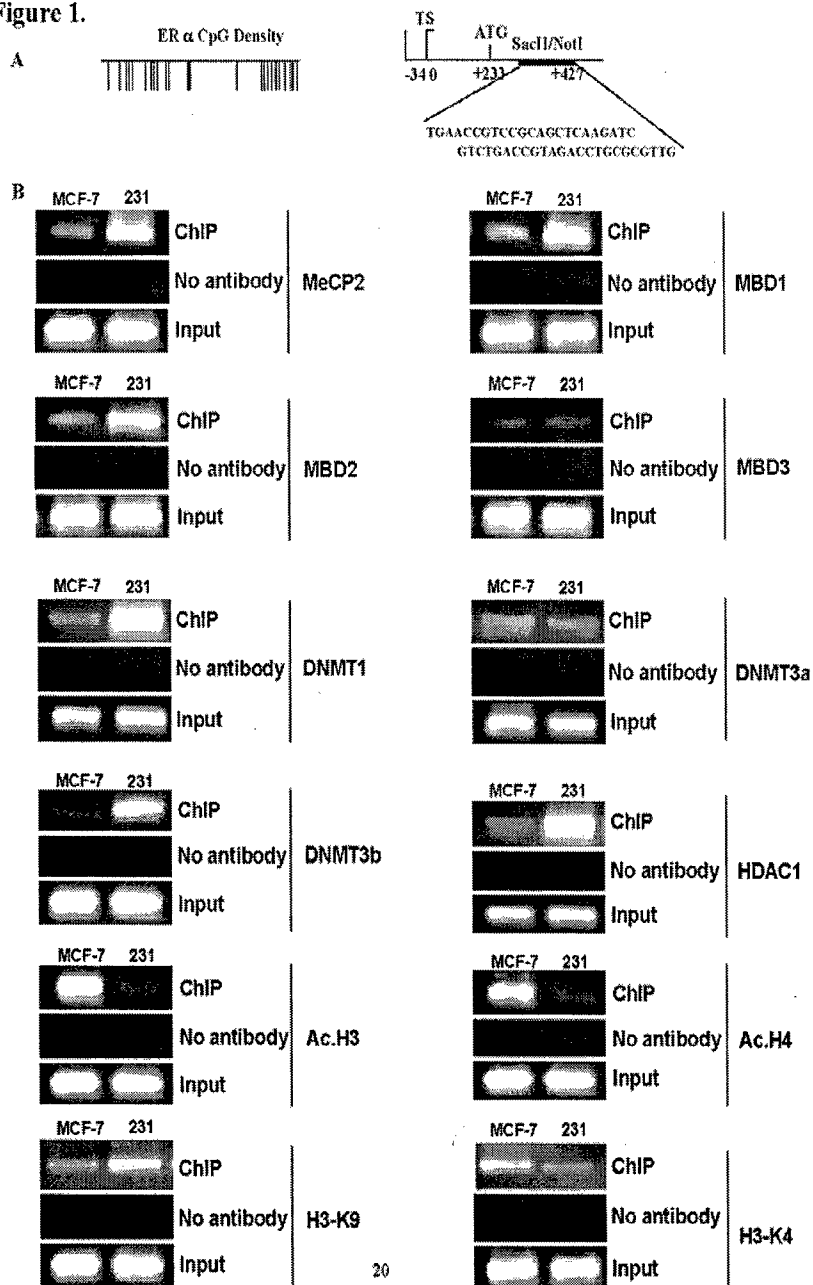
BODY

The specific objectives of this proposal are:

Task 1. **To characterize the chromatin configuration around the estrogen receptor alpha (ER) promoter in ER-negative and ER-positive human breast cancer cells (months 1-10).**
Towards this goal we studied the acetylation status of histones at the ER promoter in the active and inactive states. We also examined the methylation of histones at key lysine residues at the ER promoter. We also deciphered the key components of the repressor complex recruited at the ER promoter in activated vs repressed state. <u>We found that **ER promoter chromatin is associated with different proteins in active vs. repressed state.**</u> The ER-negative MDA-MB-231 cells with a hypermethylated ER promoter and the ER-positive MCF-7 cells with an unmethylated ER promoter were used as our model system. Using antibodies against acetylated H4, acetylated H3, methylated H3-K4, methylated H3-K9, HDAC1, MBD proteins or DNMT isoforms, formaldehyde cross-linked protein-chromatin complexes were immunoprecipitated from MCF-7 or MDA-MB-231 cells. Precipitated genomic DNA was analyzed by PCR using primers encompassing the NotI site in the ER gene, the region whose methylation is most closely associated with ER gene expression in our earlier work (Figure 1A). As shown in Figure 1B, ChIP analysis with anti MeCP2, anti MBD1, and anti MBD2 antibodies revealed that these proteins are associated with the silenced ER promoter in MDA-MB-231 cells whereas the active ER promoter in MCF-7 cells shows little or no association of these proteins. Consistent with earlier reports that MBD3 does not form specific complexes with methylated DNA, we found that MBD3 is not associated with the silenced or active ER promoter. DNMT1 and DNMT3b

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were associated with ER promoter in MDA-MB-231 but not MCF-7 cells while DNMT3a showed little association with either promoter. A significant amount of acetylated H3 and H4 was observed at the ER promoter in MCF-7 cells whereas the silenced ER promoter in MDA-MB-231 cells shows little or no association of acetylated histones. HDAC1 recruitment was observed only at the silenced chromatin in MDA-MB-231 cells. In ER negative MDA-MB-231 cells, the promoter region, which has a high level of cytosine methylation and methyl binding protein occupancy, also displayed a high level of core histone H3-K9 methylation whereas ER positive MCF-7 cells showed a higher level of H3-K4 methylation.

Figure 1.

Figure 1. Recruitment of MBDs, DNMTs, HDAC1 and histone acetylation and methylation status at the human ER CpG island. A. ER CpG island and primer sets used for ChIP assay. B. Representative results of ChIP-PCRs from MCF-7 and MDA-MB-231 human breast cancer cells with no antibody and specific antibodies (indicated on the right) are shown.

Task 2. **To examine the relationship between DNA hypermethylation and histone modifications such as acetylation, methylation and phosphorylation as regulators of ER reactivation in human breast cancer cells using pharmacological inhibitors (months 11 to 18).**

Towards accomplishing this goal we defined the optimal treatment strategies for chromatin modification by performing dose and time curves of demethylating agent and HDAC inhibitor. We used optimal drug treatments to reactivate estrogen receptor in ER-negative breast cancer cells and studied the recruitment and release of participating proteins during reactivation of ER gene.
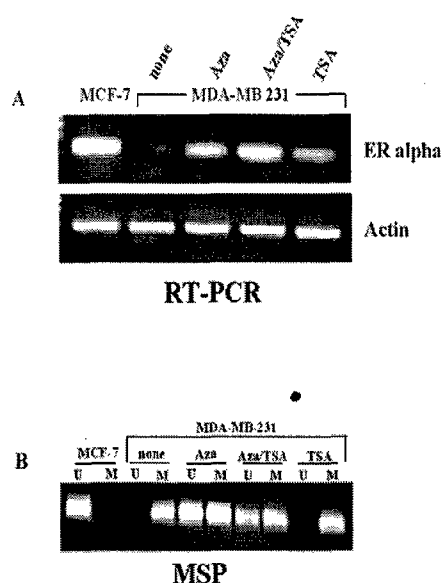
Figure 2.

Figure 2. A. ER reexpression is induced by the HDAC and DNMT inhibitors. RT-PCR analysis shows ER mRNA re-expression after treatment of MDA-MB-231 cells with 5-aza-dC (2.5 µM for 96 hours) (Aza), TSA (100 ng/ml for 12 hours), or 5-aza-dC and TSA (Aza/TSA). RT-PCR with the ER positive prototype, MCF-7 and β-actin are used as controls. **B. MSP analysis of ER CpG island.** Methylation pattern was analyzed using a previously reported primer set ER5 after 5-aza-dC and TSA treatments in MDA-MB-231 cells. ER positive MCF-7 cells and ER negative MDA-MB-231 cells were used as unmethylated and methylated controls. U- unmethylated; M- methylated.

As shown in Figure 2A, treatment of MDA-MB-231 cells with 5-aza-2'-deoxycytidine (5-aza-dC) or trichostatin A (TSA) results in reexpression of ER mRNA as detected by RT-PCR; combination therapy is more effective than either agent alone as previously described. Methylation-specific PCR (MSP) analysis also confirmed our previous findings that the ER CpG island remains methylated in MDA-MB-231 cells treated with TSA, whereas

treatment with 5-aza-dC alone or in combination with TSA partially demethylates the ER CpG island (Figure 2B). As our findings in Figure 1 clearly demonstrate specific differential recruitment of various MBD and DNMT proteins to silenced and active ER promoters, we examined the effects of inhibitors of DNMT and HDAC alone and together on proteins associated with ER promoter. <u>We found that</u> **efficient dissociation of methyl-binding proteins from the silenced ER promoter is observed after treatment with HDAC and DNMT inhibitors.**
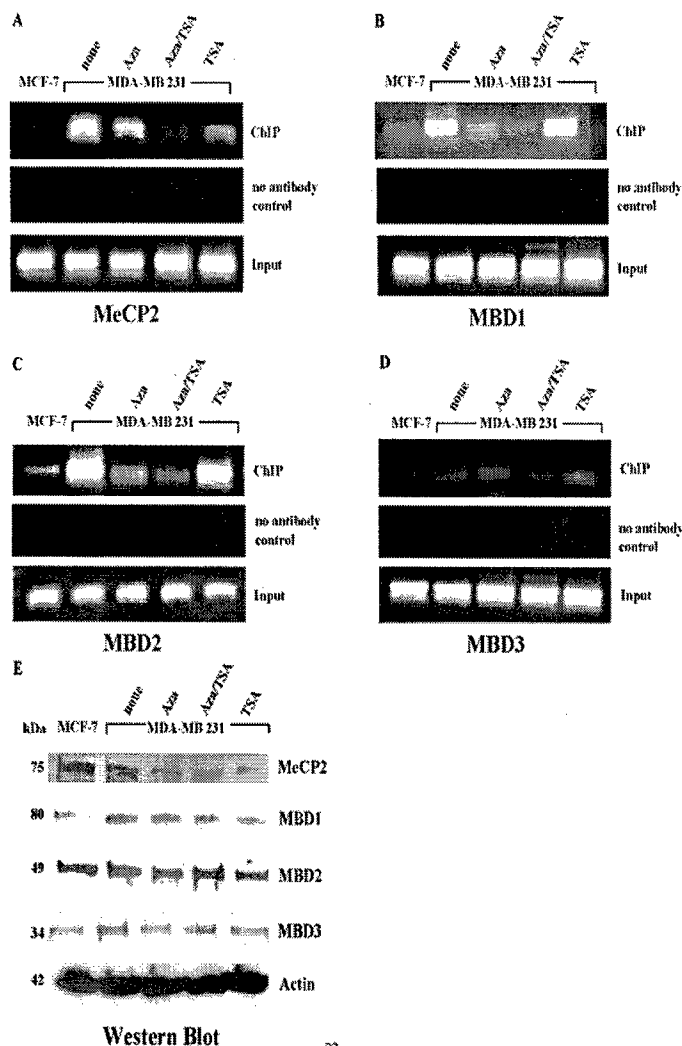
Figure 3.

Figure 3. (A-D) Profile of methyl-CpG binding proteins on ER CpG island chromatin by ChIP. Representative results of ChIP-PCRs show release of MeCP2 **(A)**, MBD1 **(B)**, and MBD2 **(C)** from the ER promoter following treatment of MDA-MB-231 cells with 5-aza-dC (AZA) and TSA. MBD3 **(D)** was not engaged on methylated or unmethylated ER chromatin. **(E) Immunoblot analysis of MBDs.** Equal amounts of protein (100 μg) from whole cell lysates from the control MCF-7 and MDA-MB-231 and MDA-MB-231 cells treated as in Figure 2 were subjected to immunoblotting with specific antibodies against MBD1, MBD2, MBD3 and MeCP2. Actin serves as protein loading control.

Treatment of MDA-MB-231 cells with 5-aza-dC or TSA alone leads to a significant reduction in MeCP2 occupancy of the ER promoter by ChIP assay (Figure 3A). The combination of 5-aza-dC and TSA further reduces MeCP2 binding in conjunction with demethylation of the ER promoter and enhanced ER expression (Figure 2A). Treatment with 5-aza-dC was associated with a similar pattern of release of MBD1 (Figure 3B) and MBD2 (Figure 3C) and this was enhanced by co-treatment with TSA. Unlike MeCP2, treatment with TSA alone did not affect association of MBD1 or MBD2 with the ER promoter in MDA-MB-231 cells. In striking contrast, there was little association of MBD3

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with the ER promoter under any circumstances (Figure 3D). **These results indicate that MeCP2, MBD1 and MBD2 (but not MBD3) are associated with the methylated inactive ER promoter in MDA-MB-231 cells and this association is dramatically reduced when the ER gene is partially demethylated and reexpressed.**

To ascertain whether these changes are simply the result of altered levels of global methyl binding protein expression in treated vs. untreated cells, immunoblot analyses were performed with antibodies specific for MeCP2, MBD1, MBD2 and MBD3 using protein lysates from MDA-MB-231 cells following treatment with 5-aza-dC, TSA or the combination (Figure 3E). None of these treatments changed global protein levels of any of the methyl binding proteins, indicating that these drugs do not substantively modulate the expression of these proteins.

Treatment of MDA-MB-231 cells with 5-aza-dC and TSA alters the association of DNMT1 and DNMT3b with ER promoter. We next determined the effects of TSA and 5-aza-dC on the association of DNMT1, DNMT3a and DNMT3b with the ER promoter using ChIP assays with specific DNMT antibodies. Treatment with 5-aza-dC but not TSA reduced the level of association of DNMT1 (Figure 4A) and DNMT3b (Figure 4C) with the ER promoter in MDA-MB-231 cells; co-treatment led to synergistic dissociation of DNMT1 and DNMT3b from the ER promoter. There was little association of DNMT3a with the ER promoter in untreated or treated cells (Figure 4B). Thus recruitment of DNMT1 and DNMT3b to methylated ER promoter decreases upon promoter demethylation.

5-aza-dC inactivates DNMTs by forming an irreversible protein-DNA complex that leads to demethylation of DNA strands followed by transcriptional activation of methylated promoter. We investigated whether treatment with DNMT or HDAC inhibitors alters the level of DNMT protein expression. Treatment of MDA-MB-231 cells with 5-aza-dC for 96 hours strikingly depleted DNMT1 and DNMT3a protein levels by Western blot (Figure 4D). Combined treatment with 5-aza-dC and TSA resulted in further depletion of both DNMT1 and DNMT3a whereas TSA alone also downregulated DNMT1 and DNMT3a slightly. The level of DNMT3b in nuclear extracts was unaltered after 5-aza-dCand/orTSA treatments. Thus reduction of global protein expression may account in part for the dissociation of DNMT1 but not DNMT3b from the ER promoter in 5-aza-dC-treated cells. These results suggest that the three DNMT isoforms might have differential sensitivity to 5-aza-dC in vivo.
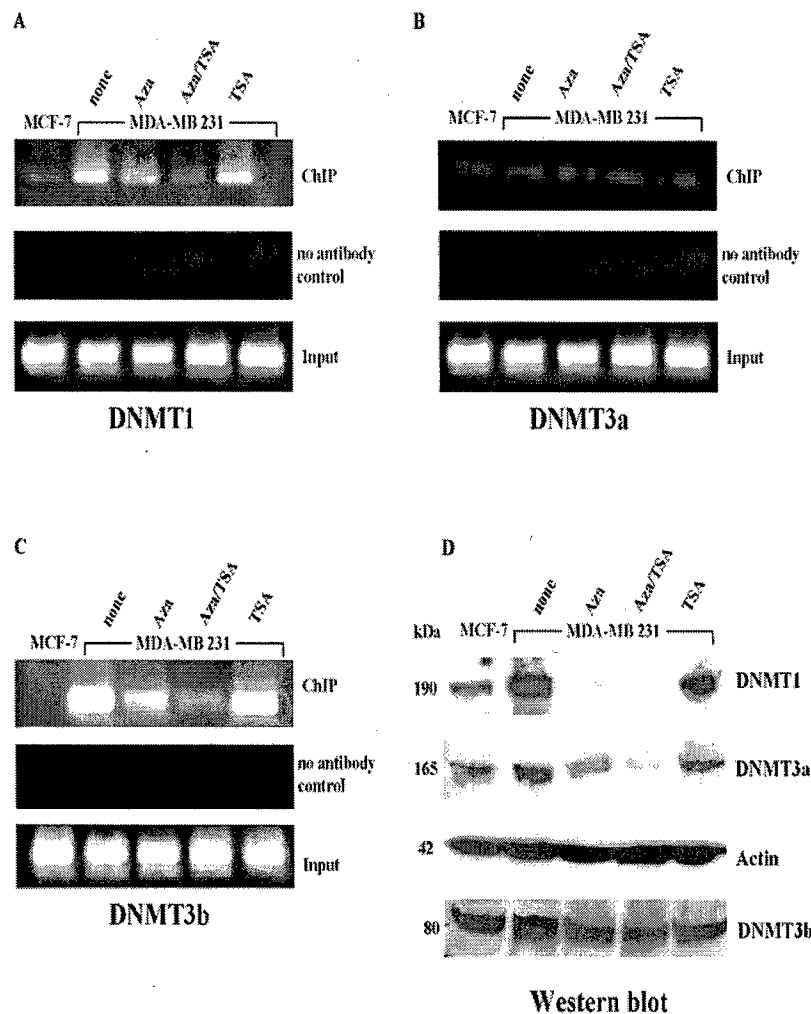
Figure 4.

Figure 4. (A-C) Profile of DNMTs on ER CpG island chromatin by ChIP. Representative ChIP-PCRs with antibodies specific for DNMT1 **(A)**, DNMT3a **(B)** and DNMT3b **(C)** from untreated and treated cells as in Figure 2 show that DNMT1 and DNMT3b dissociate from the ER promoter following treatment of MDA-MB-231 cells with 5-aza-dC (Aza) alone or in combination with TSA. DNMT3a does not associate with the ER CpG island in MCF-7 or MDA-MB-231 cells. **(D) DNMT protein expression in whole cell lysates from treated and untreated MDA-MB-231 cells.** 100μg of protein from control or treated cells were subjected to Western blot analysis with antibodies specific for DNMT1 and DNMT3a. Reprobing with anti-actin antibody shows equal loading of the protein. For DNMT3b analysis, nuclear extracts (100 μg) were prepared from control and treated cells and subjected to immunoblot analysis with anti DNMT3b antibody.

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Association of acetylated histone H3 and H4 with the ER promoter increases and HDAC1 decreases after treatment with HDAC and DNMT inhibitor. We investigated whether the observed differential recruitment of MBD and DNMT proteins to the ER promoter and their modulation by 5-aza-dC and TSA are related to altered chromatin structure. We performed ChIP assays using an antiacetylated histone H3 antibody (that recognizes histone H3 acetylated at K9 and K14) and an antiacetylated histone H4 antibody (that recognizes histone H4 acetylated at K5, K8, K12 and K16) for MDA-MB-231 cells treated with 5-aza-dC, TSA, or both. A very low level of association of acetylated histones H3 (Figure 5A) and H4 (Figure 5B) with the ER promoter was seen in MDA-MB-231 cells in contrast with MCF-7 cells. Treatment with TSA

significantly elevated the levels of association of acetylated histones H3 and H4 with the ER promoter in MDA-MB-231 cells whereas treatment with 5-aza-dC had much less effect either alone or when used in combination with TSA (Figure 5A and 5B). Not only do the MBD family proteins, MeCP2, MBD1, MBD2 and MBD3 recruit HDAC but the DNMTs, DNMT1, DNMT3a and DNMT3b, associate with the HDAC repressor complex as well. Therefore, we explored whether the association of HDAC1 with ER promoter in MDA-MB-231 cells was altered by 5-aza-dC and/or TSA treatment. ChIP assay showed that HDAC1 was associated with the ER promoter in ER-negative MDA-MB-231 cells. The association was significantly reduced by TSA or TSA/5-aza-dC treatment and was unaffected by 5-aza-dC alone (Figure 5C). These results confirm the recruitment of HDAC1 to the ER promoter in ER-negative human breast cancer cells, either directly or via interaction with various repressor complexes.

Acetylation status of the core histones H3 and H4 in MDA-MB-231 cells following treatment was also determined. Treatment of cells with TSA but not with 5-aza-dC resulted in increased global histone acetylation (Figure 5D). Western blot analyses with anti-HDAC1 antibody showed that the level of HDAC1 in MDA-MB-231 cells was not affected by treatment of cells with TSA or 5-aza-dC (Figure 5D). Collectively, these results demonstrate that TSA treatment of ER-negative human breast cancer cells results in accumulation of total acetylated histones H3 and H4 and a significant enhancement in their association with ER promoter. HDAC1 protein expression was not affected by any treatment while TSA treatment with or without 5-aza-dC led to dissociation of HDAC1 from the ER promoter.

5-aza-dC plus TSA reduces K9-methylated histone H3 and augments K4-methylated histone H3 at ER promoter. Other studies suggest a strong link between DNA methylation and H3-K9 methylation in gene repression and the organization of a heterochromatic state. We performed ChIP assays using polyclonal antibodies against K4-dimethylated H3 and K9-dimethylated H3. As shown in Figure 5E and 5F, the repressed ER state in MDA-MB-231 cells is associated with a substantial increase in methylation of K9-dimethylated H3 whereas the activated ER state in MCF-7 cells is associated with increased methylation of K4-dimethylated H3. The level of methylated H3-K9 at the ER promoter in MDA-MB-231 cells decreased after treatment with 5-aza-dC or TSA, reaching an even lower level after treatment with 5-aza-dC and TSA together (Figure 5E). In contrast, H3-K4 methylation increased at the same region after treatment with 5-aza-dC and/or TSA together (Figure 5F) in conjunction with H3 acetylation.

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Figure 5.

A

MCF-7 ┌─── MDA-MB 231 ───┐

none   Aza   Aza/TSA   TSA

ChIP

no antibody control

Input

AcH3

B

MCF-7 ┌─── MDA-MB 231 ───┐

none   Aza   Aza/TSA   TSA

ChIP

no antibody control

Input

AcH4

C

MCF-7 ┌─── MDA-MB 231 ───┐

none   Aza   Aza/TSA   TSA

ChIP

no antibody control

Input

HDAC 1

D

kDa   MCF-7 ┌─── MDA-MB 231 ───┐

none   Aza   Aza/TSA   TSA

17   AcH3

10   AcH4

65   HDAC1

Western Blot

E

MCF-7 ┌─── MDA-MB 231 ───┐

none   Aza   Aza/TSA   TSA

ChIP

no antibody control

Input

H3-K9

F

MCF-7 ┌─── MDA-MB 231 ───┐

none   Aza   Aza/TSA   TSA

ChIP

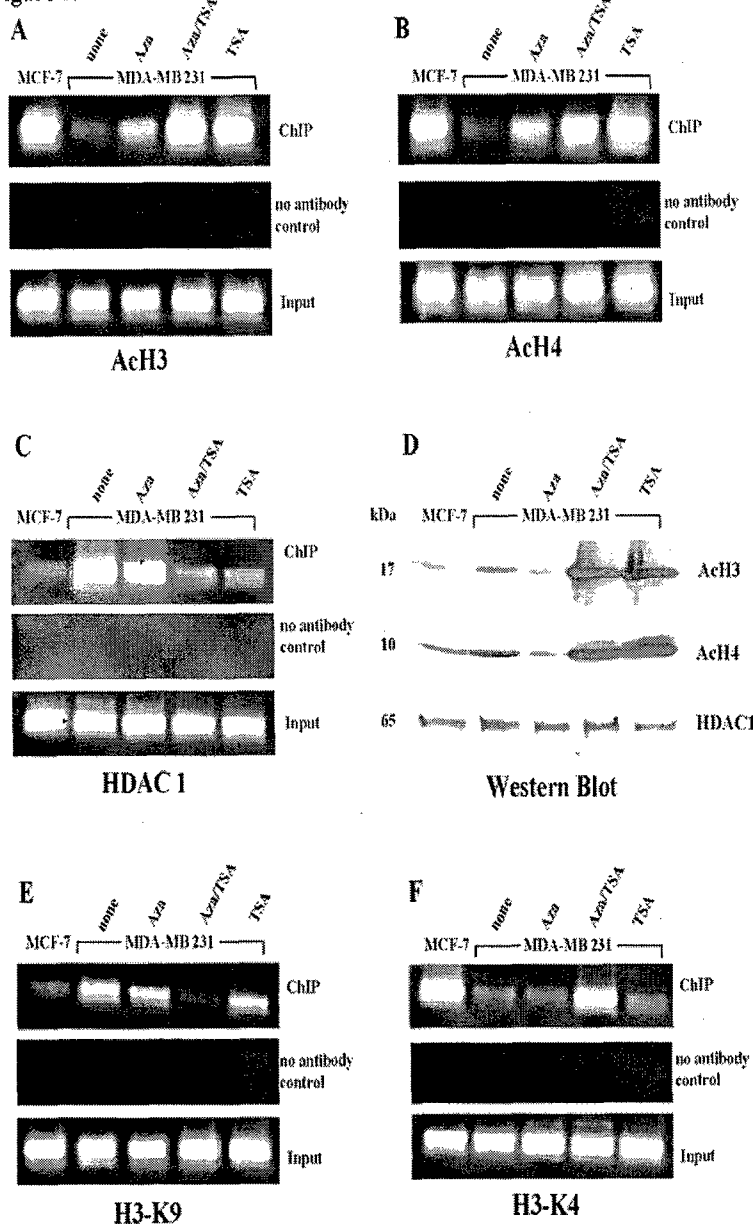no antibody control

Input

H3-K4

24

Figure 5. (A-B) Histone acetylation enrichment on active ER chromatin. ChIPs were performed with MDA-MB-231 cells treated with 5-aza-dC (Aza), TSA or 5-aza-dC and TSA together (Aza/TSA). Associated acetylated H3 and H4 histones were analyzed by PCR. **(C) Association of HDAC1 with ER promoter in MDA-MB-231 cells.** ChIP analysis with anti HDAC 1 antibody showed that HDAC1 associates with ER promoter in MDA-MB-231 cells and this association is reversed by 5-aza-dC/TSA treatment. **(D) ER chromatin is enriched in acetylated histone H3 and H4 following DNMT and HDAC inhibition.** MDA-MB-231 cells were treated with 5-aza-dC and TSA followed by histone extraction and immunoblot analysis with antibodies against acetylated histone H3 (17 kDa) and H4 (10 kDa). MCF-7 and untreated MBA-MB-231 served as controls. Western blotting using anti HDAC1 antibody showed no change in HDAC1 protein expression following the drug treatments. **(E-F) Association of methylated histones with the ER CpG island.** Chromatin was isolated from MCF-7 and MDA-MB-231 cells treated with 5-aza-dC (Aza), TSA, or the combination (Aza/TSA) and immunoprecipitations performed using specific antibodies for H3-K9 and H3-K4. Shown is a decrease in methylated H3-K9 on the ER promoter in MDA-MB-231 cells after treatments whereas the methylated H3-K4 level is increased upon treatment with 5–aza-dC and TSA together (Aza/TSA).

Task 3. To evaluate the contribution of various methyl binding proteins in ER alpha repression and reactivation in ER-negative human breast cancer cells. (months 12-24)

We are in the process of selecting and testing various SiRNA sequences for efficient silencing of various methyl binding proteins in ER-negative human breast cancer cells. We plan to silence these proteins one by one and check ER alpha repression and reactivation efficiency. We hope to finish this part of the project in the next 12 months.

KEY RESEARCH ACCOMPLISHMENTS

- Dr. Dipali Sharma, Principal Investigator of this postdoctoral award grant has been appointed to the position of Assistant Professor (RT) in Department of Hematology/Oncology, Winship Cancer Institute, Emory University, Atlanta, Georgia.

- Findings from the past one year work have been published in Molecular Endocrinology. "Dipali Sharma, Julie Blum, Xiaowei Yang, Normand Beaulieu, A. Robert Macleod and Nancy E. Davidson. Release of Methyl CpG Binding Proteins and Histone Deacetylase 1 from the Estrogen Receptor Alpha (ER) Promoter upon Reactivation in ER-negative Human Breast Cancer Cells. Mol. Endocrinol., 19(7): 1740-1751, 2005. (Reprint attached as appendix 1)

- Another part of recent findings were presented as a poster presentation in Era of Hope, Department of Defense Breast Cancer Research Program Meeting, June 8-11, 2005, Pennsylvania Convention Center, Philedelphia, Pennsylvania. Title: Restoration of tamoxifen sensitivity in ER-negative breast cancer cells.

- Also, a part of recent findings were presented as a platform presentation at the Era of Hope 2005 meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP) in "Endocrine Related Therapies" session.

- Latest findings are being communicated to "Molecular Cellular Biology" as a manuscript titled " Dipali Sharma, Neeraj K. Saxena, Frank A. Anania, Nancy E. Davidson, Paula M. Vertino. Tamoxifen-bound reactivated estrogen receptor recruits distinctive corepressor complexes. "

REPORTABLE OUTCOMES

- Dipali Sharma, Julie Blum, Xiaowei Yang, Normand Beaulieu, A. Robert Macleod and Nancy E. Davidson. Release of Methyl CpG Binding Proteins and Histone Deacetylase 1 from the Estrogen Receptor Alpha (ER) Promoter upon Reactivation in ER-negative Human Breast Cancer Cells. Mol. Endocrinol., 19(7): 1740-1751, 2005.

CONCLUSIONS

The molecular mechanisms that regulate silencing of ER in ER-negative human breast cancer cells are not well elucidated. We originally observed that DNA methylation and histone deacetylation characterized the ER promoter in ER-negative but not ER-positive human breast cancer cell lines. Treatment of these cell lines with a DNMT or HDAC inhibitor can reactivate ER mRNA expression and the combination of DNMT and HDAC inhibitor could synergistically activate functional ER mRNA and protein (4, 33).

In this work we show that the unmethylated active ER promoter in MCF-7 cells is enriched for H3 and H4 acetylation and H3-K4 methylation and shows little binding of any methyl binding protein or DNMT. In MDA-MB-231 cells, the ER promoter is silenced by DNA hypermethylation, histone hypoacetylation, H3-K9 methylation and the recruitment of MeCP2, MBD1, MBD2, DNMT1, DNMT3b and HDAC1 proteins. ER reactivation by pharmacological intervention is a complex process involving modulation of binding of various non-histone proteins and modifications of core histones such as acetylation, deacetylation and selective methylation. Treatment of MDA-MB-231 cells with the HDAC inhibitor, TSA, causes histone hyperacetylation and a low level of ER mRNA reexpression as DNMT1, DNMT3b, MeCP2, MBD1 and MBD2 are still bound to the methylated ER promoter. Treatment with the DNMT inhibitor, 5-aza-dC, also induces ER mRNA expression but it facilitates promoter demethylation along with partial dissociation of MeCP2, MBD1, MBD2, DNMT1, DNMT3b, and DNMT1. The diminished association of the ER promoter with DNMT1 may also reflect global depletion of this protein by 5-aza-dC; no such effect of 5-aza-dC on total protein for the methyl binding proteins was seen. 5-aza-dC-treated MDA-MB-231 cells also displayed a relative depletion of acetylated H3 and H4 and methylated K9 H3. Thus, treatment of MDA-MB-231 cells with 5-aza-dC or TSA leads to reexpression of ER but strikingly different protein complexes are associated with the ER promoter in each case. The combination of 5-aza-dC and TSA facilitates the release of a repressor complex containing various MBD proteins (MeCP2, MBD1 and MBD2), DNMTs (DNMT1 and DNMT3b) and HDAC1 from the ER promoter in MDA-MB-231 cells. This release was observed with concomitant enrichment of acetyl-H4, acetyl-H3, and K4-dimethylated H3 and diminished methylation at K9-H3. Thus the reactivated ER promoter in MDA-MB-231 cells treated with both drugs acquires a chromatin immunoprecipitation profile similar to that of the innately active ER promoter in MCF-7 cells.

We also demonstrate that reactivation of ER is accompanied by an increase in H3-K4 methylation and decrease in H3-K9 methylation (Figure 5). Our finding that histone H3 is methylated at the lysine 9 position at the ER promoter in MDA-MB-231 cells but not MCF-7 or drug-treated MDA-MB-231 cells is consistent with the role of H3-K9 methylation in transcription repression.

In summary the ER promoter in ER-positive MCF-7 cells is enriched for H3 and H4 acetylation and H3-K4 methylation and shows little methyl binding protein or DNMT1 association or H3-K9 methylation. In ER-negative MDA-MB-231 cells, the methylated ER promoter is associated with multiple methyl binding proteins and DNMT, H3-K9 methylation, and histone hypoacetylation. MDA-MB-231 cells treated with DNMT and HDAC inhibitors to re-express ER recapitulate the MCF-7 profile, showing a substantial increase in two euchromatic markers, H3-K4 methylation and H3 and H4 acetylation and confirming that a combination of DNMT and HDAC inhibitors can induce chromatin remodeling. The clinical importance of these observations is increasingly evident. Initial studies of DNA methylation profiles of 35 candidate epigenetically regulated genes in primary human breast cancers demonstrated significant differences in hormone receptor status between clusters of DNA methylation profiles, and methylation of the ER gene outperformed hormone receptor status as a predictor of clinical response in patients treated with tamoxifen (34). Further, much work is focused on the possibility that epigenetic changes might also be appropriate targets for treatment of malignancy. This field has been advanced by the clinical availability of the first DNMT inhibitor approved for use in humans, azacitidine, and the rapid clinical development of a number of HDAC inhibitors that are now in clinical testing (35, 36). The possibility that such agents might sensitize ER-negative human breast cancer cells to the effects of endocrine therapy has been suggested in preclinical studies such as our own showing that Hs578t cells that reexpressed ER as a consequence of 5aza-dC treatment became sensitive to the growth inhibitory effects of estrogen (37, 38). Thus our studies, that identify key molecular mechanisms for epigenetic regulation of ER expression in human breast cancer cells and provide further insight into the molecular mechanisms of action of DNMT and HDAC inhibitors alone or in combination in chromatin remodeling and modulation of gene expression, will be crucial in taking these approaches forward in breast cancer treatment.

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Appendix 1

Release of Methyl CpG Binding Proteins and Histone Deacetylase 1 from the Estrogen Receptor α (ER) Promoter upon Reactivation in ER-Negative Human Breast Cancer Cells

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Estrogen receptor α (ER) is an epigenetically regulated gene. Inhibitors of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) synergistically activate the methylated ER gene promoter in ER-negative MDA-MB-231 human breast cancer cells. Chromatin immunoprecipitation was used to examine the chromatin status and repressor complex associated with silenced ER and changes in the key regulatory factors during reactivation by inhibitors of DNMT (5-aza-2'-deoxycytidine) and HDAC (trichostatin A). The silencing of ER due to CpG hypermethylation correlates with binding of specific methyl-binding proteins, DNMTs, and HDAC proteins. Inhibition of HDAC activity by trichostatin A results in the accumulation of hyperacetylated core histones. The activation of ER gene expression by 5-aza-2'-deoxycytidine also involves the release of the repressor complex involving various methyl-binding proteins, DNMTs, and HDAC1. HDAC and DNMT inhibitors modulate histone methylation at H3-K9 and H3-K4 to form a more open chromatin structure necessary for reactivation of silenced ER transcription. Together these results impart a better understanding of molecular mechanisms of chromatin remodeling during ER reactivation by DNMT and HDAC inhibitors. These findings will aid in the application of agents targeting epigenetic changes in the treatment of breast cancer. (*Molecular Endocrinology* 19: 1740–1751, 2005)

NORMAL MAMMARY DEVELOPMENT, as well as the initiation and progression of breast cancer, are intensely influenced by hormonal factors. Breast tumors expressing estrogen receptor-α (ER) respond well to therapeutic strategies directed at the ER or its ligands, whereas those that lack ER do not (1, 2). It is imperative to understand what factors determine ER expression in breast cancer. Earlier studies have shown that the ER promoter is hypermethylated and ER mRNA is absent in some ER-negative breast cancer cells. Treatment of ER-negative breast cancer cells with DNA methyltransferase (DNMT) and/or histone deacetylase (HDAC) inhibitors leads to the reactivation of expression of ER mRNA and functional protein, underscoring the importance of DNMTs and HDACs in maintaining the repressive environment at target genes such as ER (3, 4).

The mechanisms involved in suppression of transcription of genes via hypermethylation at CpG islands and histone modifications are an area of active research (5). CpG island hypermethylation may inhibit transcription by interfering with the recruitment and function of basal transcription factors or transcriptional coactivators. Also, hypermethylation of CpG dinucleotides near the transcriptional regulatory region may initiate the recruitment of the methyl-CpG binding domain (MBD) family proteins that mediate silencing of genes via facilitation of a repressive chromatin environment (6, 7). At least five methyl-CpG binding proteins, including MeCP2, MBD1, MBD2, MBD3, and MBD4, have been identified in vertebrates (8–13). Although MeCP2, MBD1, MBD2, and MBD3 can all recruit HDAC-containing repressor complexes, distinctive features of each of these proteins have been reported (10, 14–23). Gel shift assays showed that MBD1, MBD2, and MBD4 bind specifically to a variety of DNA sequences containing methyl CpG, whereas MBD3 does not either *in vitro* or *in vivo* (19–21).

Several DNMTs that initiate methylation at position 5 of cytosines of CpG dinucleotides have been identified (24). DNMT1, the chief enzyme responsible for maintenance of mammalian DNA methylation during DNA replication using hemimethylated DNA, can also bind HDAC2 and DNMT-associated protein 1 (DMAP1) to

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mediate transcriptional repression (25). The *de novo* methylases, DNMT3a and DNMT3b, which are encoded by different genes (26, 27), can act as transcriptional repressors by using their ATRX domain to recruit HDAC1 (28, 29). Heterochromatic structure characterized by differential modifications of histones is another facet of the complex machinery influencing repression. Amino-terminal tails of the core histones undergo modifications such as acetylation at lysine, methylation at lysine or arginine, and phosphorylation at serine to evolve a histone code for transcriptional activation and repression (30, 31). These posttranslational modifications modulate the chromatin structure by altering the electrostatic interactions between histone proteins and DNA and also by modifying the recruitment of various nonhistone proteins such as coactivators and corepressors to chromatin.

The findings that CpG methylation of the ER promoter results in transcriptional silencing (32) and inhibition of HDAC and/or DNMT activity reactivates ER (3, 4) support a model in which methyl-CpG binding proteins, DNMTs, and HDACs might be involved in transcriptional control of ER. Chromatin immunoprecipitation (ChIP) was used to monitor the epigenetic determinants of ER chromatin remodeling associated with ER expression.

RESULTS

ER Promoter Chromatin Is Associated with Different Proteins in Active *vs.* Repressed State

The ER-negative MDA-MB-231 cells with a hypermethylated ER promoter and the ER-positive MCF-7 cells with an unmethylated ER promoter were used as our model system. Using antibodies against acetylated H4, acetylated H3, methylated H3-K4, methylated H3-K9, HDAC1, various MBD proteins, or DNMT isoforms, formaldehyde cross-linked protein-chromatin complexes were immunoprecipitated from MCF-7 cells or MDA-MB-231 cells. Precipitated genomic DNA was analyzed by PCR using primers encompassing the *Not*I site in the ER gene, the region where methylation is most closely associated with ER gene expression in our earlier work (Fig. 1A). As shown in Fig. 1B, ChIP analysis with anti-MeCP2, anti-MBD1, and anti-MBD2 antibodies revealed that these proteins are associated with the silenced ER promoter in MDA-MB-231 breast cancer cells, whereas the active ER promoter in MCF-7 cells shows little association of these proteins. Consistent with earlier reports that MBD3 does not form specific complexes with methylated DNA (19–21), we found that MBD3 is not associated with the silenced or active ER promoter. Moreover, DNMT1 and DNMT3b were associated with ER promoter in MDA-MB-231 but not MCF-7 cells, whereas DNMT3a showed little association with either promoter. A significant amount of acetylated H3 and H4 was observed at the ER promoter in MCF-7 cells,

as evidenced by ChIP analyses using antiacetylated histone H3 and H4 antibodies, whereas the silenced ER promoter in MDA-MB-231 cells showed little or no association of acetylated histones. HDAC1 recruitment was observed only at the silenced chromatin in MDA-MB-231 cells. In ER-negative MDA-MB-231 cells, the promoter region, which has a high level of cytosine methylation and methyl binding protein occupancy, also displayed a high level of core histone H3-K9 methylation, whereas ER-positive MCF-7 cells showed a higher level of H3-K4 methylation.

Efficient Dissociation of Methyl-Binding Proteins from the Silenced ER Promoter Is Observed after Treatment with 5-aza-2′-Deoxycytidine (5-aza-dC) and Trichostatin A (TSA)

As shown in Fig. 2A, treatment of MDA-MB-231 cells with 5-aza-dC or TSA results in reexpression of ER mRNA as detected by RT-PCR; combination therapy is more effective than either agent alone as previously described (4). Methylation-specific PCR (MSP) analysis also confirmed our previous findings (4) that the ER CpG island remains methylated in MDA-MB-231 cells treated with TSA, whereas treatment with 5-aza-dC alone or in combination with TSA partially demethylates the ER CpG island (Fig. 2B). As our findings in Fig. 1 clearly demonstrate specific differential recruitment of various MBD and DNMT proteins to silenced and active ER promoters in ER-negative and -positive human breast cancer cells, respectively, we examined the effects of inhibitors of DNMT and HDAC alone and together on proteins associated with ER promoter.

Treatment of MDA-MB-231 cells with 5-aza-dC or TSA alone leads to a significant reduction in MeCP2 occupancy of the ER promoter by ChIP assay (Fig. 3A). The combination of 5-aza-dC and TSA further reduces MeCP2 binding in conjunction with demethylation of the ER promoter and enhanced ER expression as shown in Fig. 2A. Treatment with 5-aza-dC was associated with a similar pattern of release of MBD1 (Fig. 3B) and MBD2 (Fig. 3C), and this was enhanced by cotreatment with TSA. Unlike MeCP2, treatment with TSA alone did not affect association of MBD1 or MBD2 with the ER promoter in MDA-MB-231 cells. In striking contrast, there was little association of MBD3 with the ER promoter under any circumstances (Fig. 3D). Taken together, these results indicate that MeCP2, MBD1, and MBD2 (but not MBD3) are indeed associated with the methylated inactive ER promoter in MDA-MB-231 cells, and this association is dramatically reduced when the ER gene is partially demethylated and reexpressed.

To ascertain whether these changes are simply the result of altered levels of global methyl-binding protein expression in treated *vs.* untreated cells, immunoblot analyses were performed with antibodies specific for MeCP2, MBD1, MBD2, and MBD3 using protein lysates from MDA-MB-231 cells after treatment with 5-aza-dC, TSA, or the combination (Fig. 3E). None of
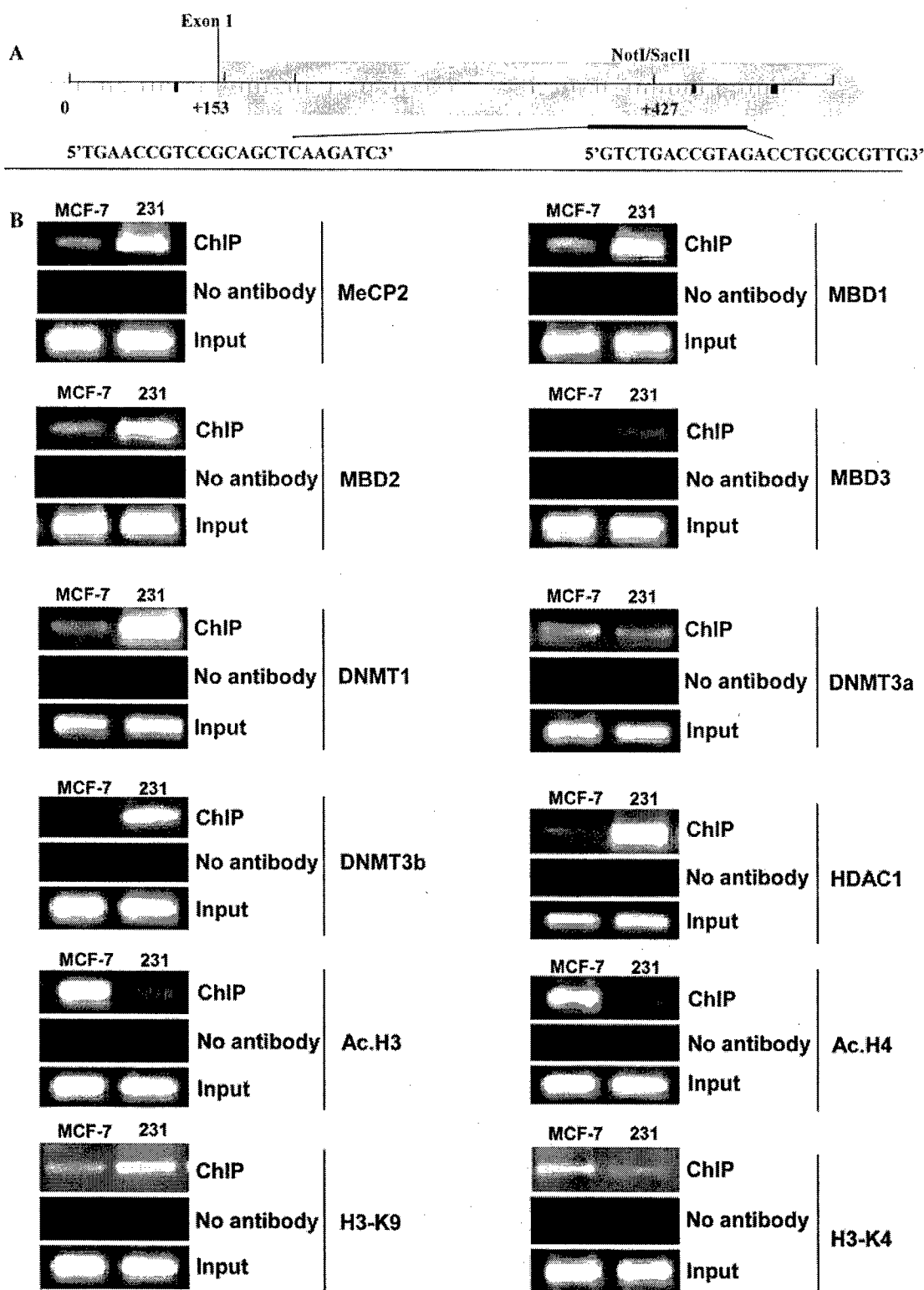
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Fig. 1. Recruitment of MBDs, DNMTs, HDAC1, and Histone Acetylation and Methylation Status at the Human ER CpG Island
A, ER CpG island and primer sets used for ChIP assay. B, Cross-linked chromatin prepared from ER-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cells was immunoprecipitated with antibodies indicated on the *right*. The immunoprecipitates were subjected to PCR analysis using primer pairs spanning the *Not*I site on the ERα promoter CpG island (depicted schematically in panel A). Aliquots of chromatin taken before immunoprecipitation were used as input controls, whereas chromatin aliquots eluted from immunoprecipitations lacking antibody were used as no antibody controls. MeCP2, Methyl-CpG protein 2; Ac.H3, acetylated H3 histone; Ac.H4, acetylated H4 histone.
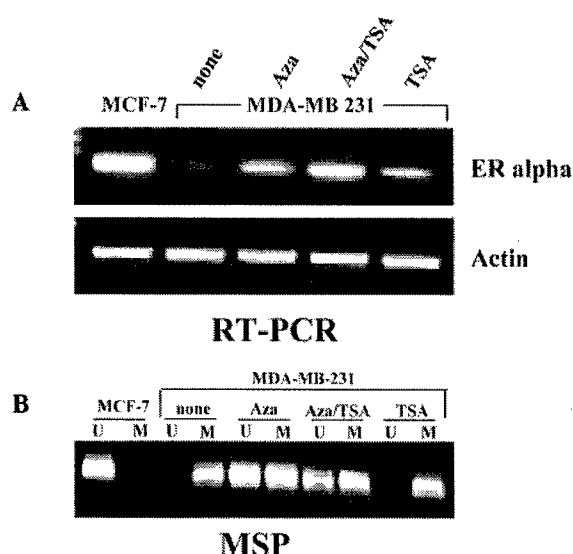
Appendix 1

Fig. 2. ER mRNA Expression and ER Gene Methylation Status after Aza and/or TSA Treatment

A, ER reexpression is induced by the HDAC and DNMT inhibitors. RT-PCR analysis shows ER mRNA reexpression after treatment of MDA-MB-231 cells with 5-aza-dC (2.5 μM for 96 h) (Aza), TSA (100 ng/ml for 12 h), or 5-aza-dC and TSA (Aza/TSA). The ER-positive prototype, MCF-7, was used as an RT-PCR-positive control. The RT-PCR product for β-actin provides a control for the amount of intact RNA used in the reactions. B, MSP analysis of ER CpG island. Methylation pattern was analyzed using a previously reported primer set ER5 (32) after 5-aza-dC and TSA treatments in MDA-MB-231 cells. ER-positive MCF-7 cells were used as an unmethylated control, whereas untreated ER-negative MDA-MB-231 cells were used as a methylated control. U, Unmethylated; M, methylated.

these treatments changed global protein levels of any of the methyl-binding proteins, indicating that these drugs do not modulate the expression of these proteins in a substantive way.

Treatment of MDA-MB-231 cells with 5-aza-dC and TSA Alters the Association of DNMT1 and DNMT3b with ER Promoter

We next determined the effects of the HDAC inhibitor, TSA, and the DNMT inhibitor, 5-aza-dC, on the association of DNMT1, DNMT3a, and DNMT3b with the ER promoter using ChIP assays with specific DNMT antibodies. Treatment with 5-aza-dC, but not TSA, reduced the level of association of DNMT1 (Fig. 4A) and DNMT3b (Fig. 4C) with the ER promoter in MDA-MB-231 cells; cotreatment led to synergistic dissociation of DNMT1 and DNMT3b from the ER promoter. There was little association of DNMT3a with the ER promoter in untreated or treated cells (Fig. 4B). Thus, recruitment of DNMT1 and DNMT3b to methylated ER promoter decreases upon demethylation of the promoter.

5-aza-dC inactivates DNMTs by forming an irreversible protein-DNA complex that leads to demethylation

of DNA strands followed by transcriptional activation of methylated promoter. We investigated whether treatment with DNMT or HDAC inhibitors alters the level of DNMT protein expression. Treatment of MDA-MB-231 cells with 5-aza-dC for 96 h strikingly depleted DNMT1 and DNMT3a protein levels by Western blot (Fig. 4D). Combined treatment with 5-aza-dC and TSA resulted in further depletion of both DNMT1 and DNMT3a, whereas TSA alone also down-regulated DNMT1 and DNMT3a slightly. The level of DNMT3b in nuclear extracts was unaltered after 5-aza-dC and/or TSA treatments. Thus, reduction of global protein expression may account, in part, for the dissociation of DNMT1 but not DNMT3b from the ER promoter in 5-aza-dC-treated cells. These results suggest that the three DNMT isoforms might have differential sensitivity to 5-aza-dC *in vivo*, a finding previously reported in HCT116 colon cancer cells (36).

Association of Acetylated Histone H3 and H4 with the ER Promoter Increases and HDAC1 Decreases after Treatment with HDAC and DNMT Inhibitor

We investigated whether the observed differential recruitment of MBD and DNMT proteins to the ER promoter and their modulation by 5-aza-dC and TSA are related to altered chromatin structure. Generally, transcriptionally inactive methylated promoters are associated with hypoacetylated histones, whereas active promoters are associated with hyperacetylated histones. We wished to determine whether the enhanced reexpression of ER in MDA-MB-231 cells after treatment with 5-aza-dC and TSA is due to localized hyperacetylation of histones at ER promoter. We performed ChIP assays using an antiacetylated histone H3 antibody (that recognizes histone H3 acetylated at K9 and K14) and an antiacetylated histone H4 antibody (that recognizes histone H4 acetylated at K5, K8, K12, and K16) using MDA-MB-231 cells treated with 5-aza-dC, TSA, or the combination. A very low level of association of acetylated histone H3 (Fig. 5A) and acetylated histone H4 (Fig. 5B) with the ER promoter was seen in MDA-MB-231 cells in contrast with MCF-7 cells. Treatment with TSA significantly elevated the levels of association of acetylated histone H3 and acetylated histone H4 with the ER promoter in MDA-MB-231 cells, whereas treatment with 5-aza-dC had much less effect either alone or when used in combination with TSA (Fig. 5, A and B).

Not only do the MBD family proteins (MeCP2, MBD1, MBD2, and MBD3) recruit HDAC (10, 21–23) but the DNMTs [DNMT1 (25), DNMT3a, and DNMT3b (26, 27)] associate with the HDAC repressor complex as well (28, 29). Therefore, we explored whether the association of HDAC1 with ER promoter in MDA-MB-231 cells was altered by 5-aza-dC and/or TSA treatment. ChIP assay performed with specific HDAC1 antibody showed that HDAC1 was associated with the ER promoter in ER-negative MDA-MB-231 cells. The
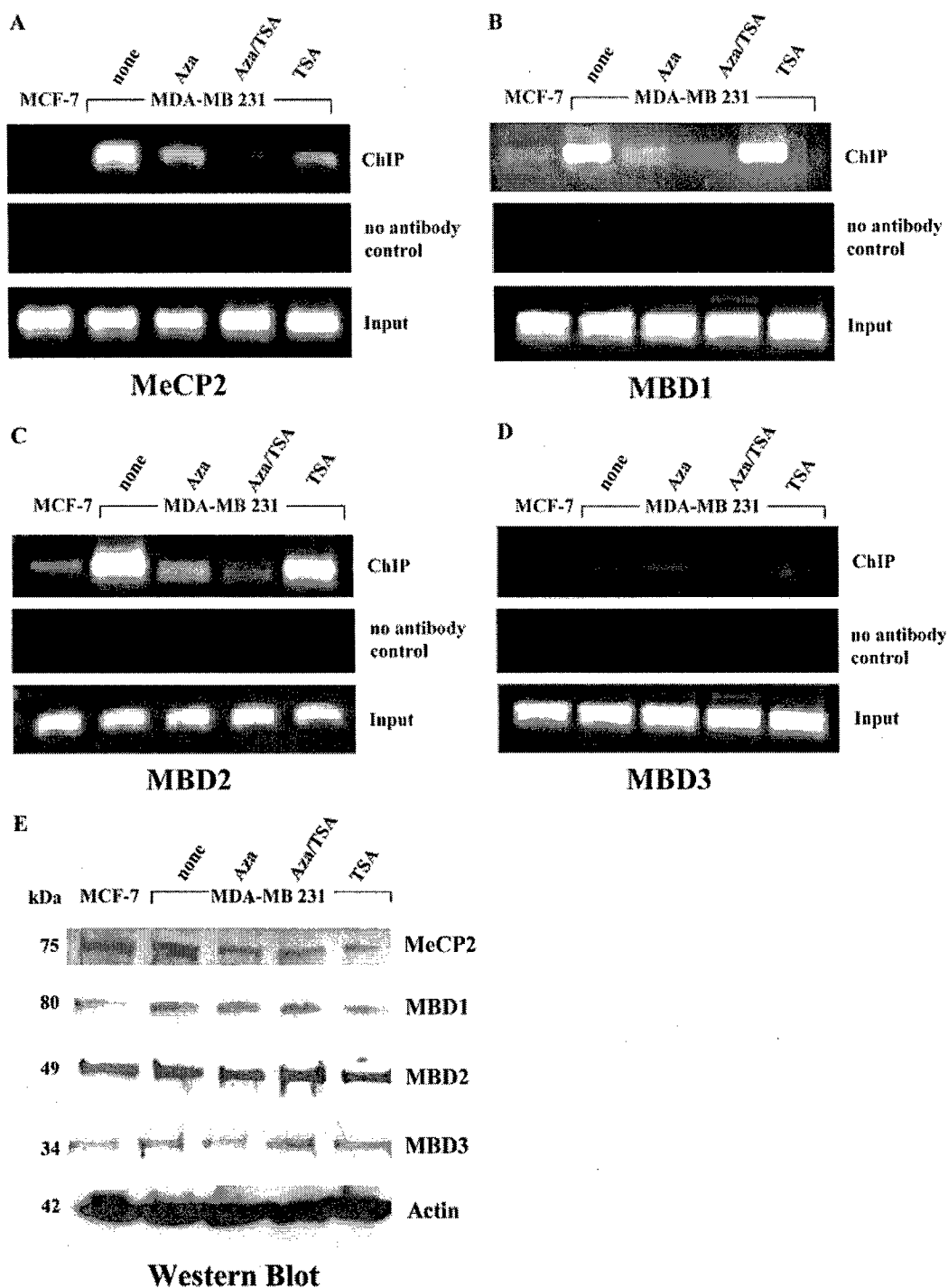
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Fig. 3. Association of Methyl-Binding Proteins at the ER Promoter after Aza and/or TSA Treatment

A–D, Profile of methyl-CpG binding proteins on ER CpG island chromatin by ChIP. The formaldehyde-cross-linked chromatin isolated from 10^7 MCF-7 or MDA-MB-231 cells (untreated or treated as in Fig. 2) was precleared with protein A agarose/salmon sperm DNA beads for 1 h at 4 C. The supernatant was incubated with 5 μg of specific antibodies overnight at 4 C. The immune complexes were pulled down with protein A agarose/salmon sperm DNA beads and washed extensively as described in *Materials and Methods*, and cross-linking was reversed. The purified DNA was analyzed by PCR using primers spanning the *Not*I site in ER CpG island. Release of MeCP2 (A), MBD1 (B), and MBD2 (C) from the ER promoter after treatment of MDA-MB-231 cells with the demethylating agent 5-aza-dC (Aza) and the HDAC1 inhibitor TSA was observed. MBD3 (D) was not engaged on methylated or unmethylated ER chromatin. E, Immunoblot analysis of MBDs. Equal amounts of protein (100 μg) from whole-cell lysates from the control MCF-7 and MDA-MB-231 and MDA-MB-231 cells treated as in Fig. 2 were separated by SDS-PAGE and subjected to Western blot analysis with specific antibodies against MBD1, MBD2, MBD3, and MeCP2. Equivalence of protein loading was demonstrated by immunoblotting with antiactin antibody.

Appendix 1

A

DNMT1

B

DNMT3a

C
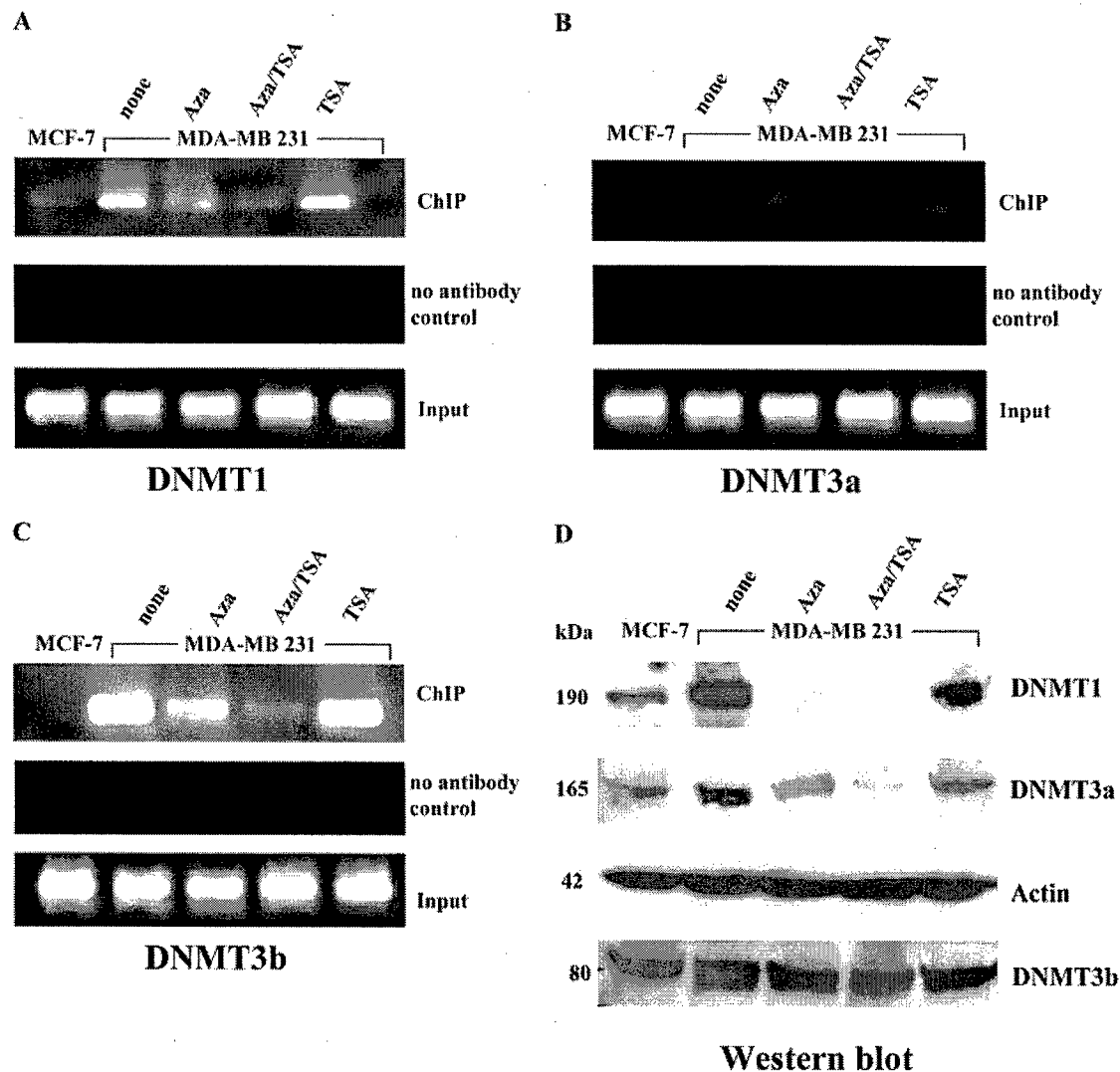

DNMT3b

D

Western blot

Fig. 4. Association of DNA Methyl-Transferases at the ER Promoter after Aza and/or TSA Treatment

A–C, Profile of DNMTs on ER CpG island chromatin by ChIP. Formaldehyde cross-linked chromatin was immunoprecipitated with antibodies specific for DNMT1 (A), DNMT3a (B), and DNMT3b (C) from untreated and treated cells as in Fig. 2. The purified DNA was amplified for the ER promoter as in Fig. 3. DNMT1 and DNMT3b dissociate from the ER promoter CpG island after treatment of MDA-MB-231 cells with 5-aza-dC (Aza) alone or in combination with TSA. DNMT3a does not associate with the ER CpG island in MCF-7 or MDA-MB-231 cells. Controls show input genomic DNA before the addition of antibody and eluants from no antibody immunoprecipitations. D, DNMT protein expression in the whole-cell lysates from treated and untreated MDA-MB-231 cells. Identical amounts (100 μg) of protein from control or treated cells were separated by SDS-PAGE on a 8% acrylamide gel, transferred to a nitrocellulose membrane, and subjected to Western blot analysis with antibodies specific for DNMT1 and DNMT3a. The membranes were reprobed with antiactin antibody to show equal loading of the protein. For DNMT3b analysis, nuclear extracts (100 μg) were prepared from control and treated cells and subjected to immunoblot analysis with anti-DNMT3b antibody.

association was significantly reduced by TSA or TSA/ 5-aza-dC treatment and was unaffected by 5-aza-dC alone (Fig. 5C). These results confirm the recruitment of HDAC1 to the ER promoter in ER-negative human breast cancer cells, either directly or via interaction with various repressor complexes.

Acetylation status of the core histones H3 and H4 in MDA-MB-231 cells after treatment was also determined. Histones were purified from the nuclei of control and treated cells and analyzed by immunoblotting.

As shown in Fig. 5D, treatment of cells with TSA, but not with 5-aza-dC, resulted in increased global histone acetylation. These results clearly demonstrate that HDAC inhibition results in the accumulation of hyperacetylated histones, leading to more open chromatin structure, whereas the DNMT inhibitor was ineffective in this regard. Next, we investigated the effects of TSA and 5-aza-dC on the expression of HDAC1 protein. Western blot analyses with anti-HDAC1 antibody showed that the level of HDAC1 in MDA-MB-231 cells
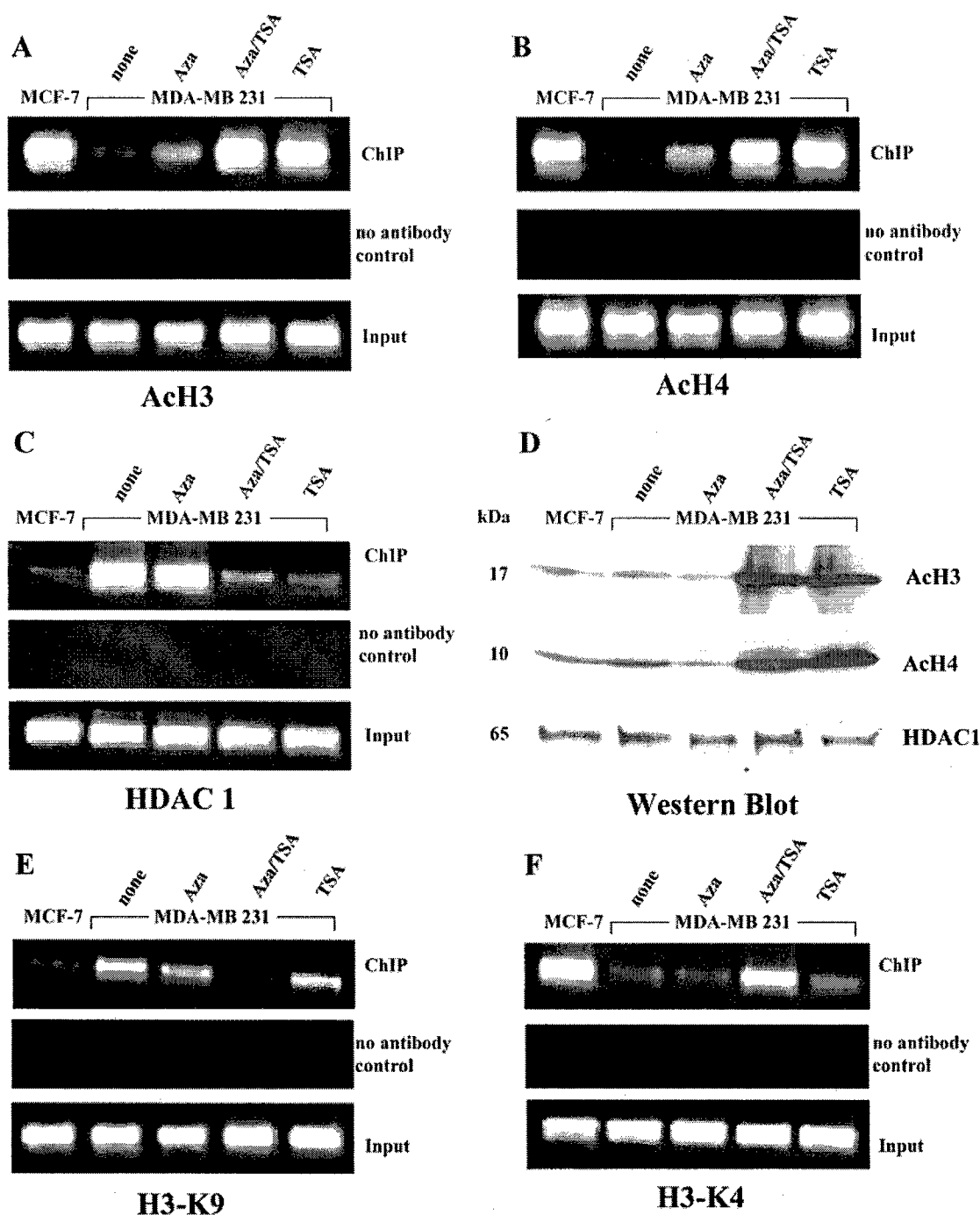
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Fig. 5. Association of Modified Histones and HDAC1 at the ER Promoter after Aza and/or TSA Treatment

A and B, Histone acetylation enrichment on active ER chromatin. ChIPs were performed with MDA-MB-231 cells treated with 5-aza-dC (Aza), TSA, or 5-aza-dC and TSA together (Aza/TSA). Associated acetylated H3 and H4 (AcH3, AcH4) histones were analyzed by PCR. C, Association of HDAC1 with ER promoter in MDA-MB-231 cells. ChIP analysis with anti-HDAC 1 antibody showed that HDAC1 associates with ER promoter in MDA-MB-231 cells, and this association is reversed by 5-aza-dC/TSA treatment. D, ER chromatin is enriched in acetylated histone H3 and H4 after DNMT and HDAC inhibition. MDA-MB-231 cells were treated with 5-aza-dC and TSA, nuclei were isolated, and histones were extracted. Equal amounts of protein (30 μg) were run on a 12% SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis with antibodies against acetylated histone H3 (17 kDa) and H4 (10 kDa). MCF-7 and untreated MBA-MB-231 served as controls. Western blotting using anti-HDAC1 antibody showed no change in HDAC1 protein expression after the drug treatments. E and F, Association of methylated H3-K9 and H3-K4 with the ER CpG island. Soluble chromatin was isolated from MCF-7 and MDA-MB-231 cells treated with 5-aza-dC (Aza), TSA, or the combination (Aza/TSA). Immunoprecipitations were performed using specific antibodies for H3-K9 and H3-K4. Shown is a decrease in methylated H3-K9 on the ER promoter in MDA-MB-231 cells after treatments, whereas the methylated H3-K4 level is increased upon treatment with 5-aza-dC and TSA together (Aza/TSA).

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was not affected by treatment of cells with TSA or 5-aza-dC (Fig. 5D). Collectively, these results demonstrate that treatment of ER-negative human breast cancer cells with TSA results in accumulation of total acetylated histones H3 and H4 as well as a significant enhancement in their association with ER promoter. HDAC1 protein expression was not affected by any treatment whereas TSA treatment with or without 5-aza-dC led to dissociation of HDAC1 from the ER promoter.

5-aza-dC Plus TSA Reduces K9-Methylated Histone H3 and Augments K4-Methylated Histone H3 at ER Promoter

Other studies suggest a strong link between DNA methylation and H3-K9 methylation in gene repression and the organization of a heterochromatic state (5, 43, 44). We performed ChIP assays using polyclonal antibodies against K4-dimethylated H3 and K9-dimethylated H3 in MCF-7 and MDA-MB-231 breast cancer cells. As shown in Fig. 5, E and F, the repressed ER state in MDA-MB-231 cells is associated with a substantial increase in methylation of K9-dimethylated H3, whereas the activated ER state in MCF-7 cells is associated with increased methylation of K4-dimethylated H3. Because our previous experiments indicated that 5-aza-dC and TSA reactivate silenced ER in MDA-MB-231 cells by altering the chromatin structure, we reasoned that these agents might change the methylation status of histone H3 during the reactivation process. The level of methylated H3-K9 at the ER promoter decreased after treatment with 5-aza-dC or TSA, reaching an even lower level after treatment with 5-aza-dC and TSA together (Fig. 5E). In contrast, H3-K4 methylation increased at the same region after 5-aza-dC and/or TSA together (Fig. 5F) in conjunction with H3 acetylation (Fig. 5A). We conclude that reactivation of ER gene in MDA-MB-231 involves chromatin remodeling of the promoter where the heterochromatic imprint was reversed and replaced with a euchromatic imprint.

DISCUSSION

The molecular mechanisms that regulate silencing of ER in ER-negative human breast cancer cells are not well elucidated. We originally observed that DNA methylation and histone deacetylation characterized the ER promoter in ER-negative, but not ER-positive, human breast cancer cell lines. Treatment of these cell lines with a DNMT or HDAC inhibitor can reactivate ER mRNA expression, and the combination of DNMT and HDAC inhibitor could synergistically activate functional ER mRNA and protein (4, 45).

Here we show that the unmethylated active ER promoter in MCF-7 cells is enriched for H3 and H4 acetylation and H3-K4 methylation and shows little binding

of any methyl-binding protein or DNMT. In MDA-MB-231 cells, the ER promoter is silenced by DNA hypermethylation, histone hypoacetylation, H3-K9 methylation and the recruitment of MeCP2, MBD1, MBD2, DNMT1, DNMT3b, and HDAC1 proteins. ER reactivation by pharmacological intervention is a complex process involving modulation of binding of various non-histone proteins and modifications of core histones such as acetylation, deacetylation and selective methylation. Treatment of MDA-MB-231 cells with the HDAC inhibitor, TSA, causes histone hyperacetylation and a low level of ER mRNA reexpression as DNMT1, DNMT3b, MeCP2, MBD1, and MBD2 are still bound to the methylated ER promoter. Treatment with the DNMT inhibitor, 5-aza-dC, also induces ER mRNA expression, but it facilitates promoter demethylation along with partial dissociation of MeCP2, MBD1, MBD2, DNMT1, DNMT3b, and DNMT1. The diminished association of the ER promoter with DNMT1 may also reflect global depletion of this protein by 5-aza-dC; no such effect of 5-aza-dC on total protein for the methyl-binding proteins was seen. 5-aza-dC-treated MDA-MB-231 cells also displayed a relative depletion of acetylated H3 and H4 and methylated K9 H3. Thus, treatment of MDA-MB-231 cells with 5-aza-dC or TSA leads to reexpression of ER, but strikingly different protein complexes are associated with the ER promoter in each case. The combination of 5-aza-dC and TSA facilitates the release of a repressor complex containing various MBD proteins (MeCP2, MBD1, and MBD2), DNMTs (DNMT1 and DNMT3b), and HDAC1 from the ER promoter in MDA-MB-231 cells. This release was observed with concomitant enrichment of acetyl-H4, acetyl-H3, and K4-dimethylated H3 and diminished methylation at K9-H3. Thus the reactivated ER promoter in MDA-MB-231 cells treated with both drugs acquires a ChIP profile similar to that of the innately active ER promoter in MCF-7 cells.

Similar studies have been carried out with other epigenetically regulated gene promoters. Studies of human multidrug resistance (MDR1) gene regulation have shown that histone acetylation and dissociation of MeCP2 and HDAC1 mark the activation of MDR1 gene upon treatment with TSA and 5-aza-dC whereas other MBD proteins such as MBD1, MBD2, MBD3, and MBD4 are not involved in MDR1 activation in T cell leukemia cell lines (15). In lymphosarcoma cells, the activation of the metallothionein I (MT-I) promoter by 5-aza-dC and TSA is linked with dissociation of MeCP2 and absence of effect on MBD1 or MBD3 (33). In both hepatocellular carcinoma and MCF-7 human breast cancer cells, MBD2, but not MeCP2, has been implicated in glutathione *S*-transferase P1 (GSTP1) silencing at the hypermethylated GSTP1 CpG island (46, 47). A gene-specific profile of MBD association with other genes including the Ras association domain family 1A gene (RASSF1A), the GSTP1 gene, the retinoic acid receptor β 2 (RARβ2) gene, the breast cancer 1 (BRCA1) gene, the O^6-methylguanine-DNA

Appendix 1

methyltransferase (MGMT) gene and the mutL-homolog 1 (MLH1) gene in MCF7 and MDA-MB-231 cells has also been determined (48). This genome-wide analysis revealed that some methylated sequences associate with only one MBD whereas others associate with several or all of them. Thus the nature of the repressor complex appears to be gene specific and is influenced by several variables such as promoter methylation, histone modification patterns, and transcription factor requirements.

We also demonstrate that reactivation of ER is accompanied by an increase in H3-K4 methylation and decrease in H3-K9 methylation (Fig. 5). Whereas the role of histone acetylation in transcriptional regulation is well recognized, recent studies have underlined the importance of histone methylation in gene expression (49). The effect of methylation of lysine residues on the N-terminal tails of histones appears to be site specific. The identification of SUV39H1 and its yeast homolog, Clr4, as the H3-K9-specific histone methyltransferase provides the first direct connection between H3-K9 methylation and heterochromatin gene silencing (50). A recent study reported that multimolecular complexes containing pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 mediate transcription of ER in breast cancer cells (51). Our finding that histone H3 is methylated at the lysine 9 position at the ER promoter in MDA-MB-231 cells, but not MCF-7 or drug-treated MDA-MB-231 cells, is consistent with the role of H3-K9 methylation in transcription repression. H3-K9 methylation has been shown to create a binding site for HP1 in the N-terminal tail of histone H3 (52); thus, H3-K9 methylation might facilitate ER repression in MDA-MB-231 cells by recruiting HP1. Another mechanism for ER repression in ER-negative cells can be through MBD1 as MBD1 interacts with the SUV39H1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression (53). Future work will test whether HP1 is indeed recruited to the ER promoter region during repression. Also, MeCP2 has been shown to associate with histone methyltransferase activity *in vivo*, and this activity is directed against lysine 9 of histone H3 (54). Our results show that MeCP2 is associated with ER promoter in MDA-MB-231 cells where histone H3 is methylated at the lysine 9 position. 5-aza-dC treatment induces release of MBD proteins, reduction in H3-K9 methylation, and enhanced H3 acetylation and H3-K4 methylation. Similar results have been shown for the MDR1 gene (15), the silenced loci p14ARF/p16INK4α in T24 bladder cancer cells (55), and at several genes in MCF7 and MDA-MB-231 cells (48).

In summary, the ER promoter in ER-positive MCF-7 cells is enriched for H3 and H4 acetylation and H3-K4 methylation and shows little methyl-binding protein or DNMT1 association or H3-K9 methylation. In ER-negative MDA-MB-231 cells, the methylated ER promoter is associated with multiple methyl-binding proteins and DNMT, H3-K9 methylation, and histone hypoacetylation. MDA-MB-231 cells treated with DNMT and HDAC inhibitors to reexpress ER recapitulate the

MCF-7 profile, showing a substantial increase in two euchromatic markers, H3-K4 methylation and H3 and H4 acetylation, and confirming that a combination of DNMT and HDAC inhibitors can induce chromatin remodeling. The clinical importance of these observations is increasingly evident. Initial studies of DNA methylation profiles of 35 candidate epigenetically regulated genes in primary human breast cancers demonstrated significant differences in hormone receptor status between clusters of DNA methylation profiles, and methylation of the ER gene outperformed hormone receptor status as a predictor of clinical response in patients treated with tamoxifen (56). Further, much work is focused on the possibility that epigenetic changes might also be appropriate targets for treatment of malignancy. This field has been advanced by the clinical availability of the first DNMT inhibitor approved for use in humans, azacitidine, and the rapid clinical development of a number of HDAC inhibitors that are now in clinical testing (57, 58). The possibility that such agents might sensitize ER-negative human breast cancer cells to the effects of endocrine therapy has been suggested in preclinical studies, such as our own, showing that Hs578t cells that reexpressed ER as a consequence of 5aza-dC treatment became sensitive to the growth-inhibitory effects of estrogen (34). Thus, our studies, which identify key molecular mechanisms for epigenetic regulation of ER expression in human breast cancer cells and provide further insight into the molecular mechanisms of action of DNMT and HDAC inhibitors alone or in combination in chromatin remodeling and modulation of gene expression, will be crucial in taking these approaches forward in breast cancer treatment.

MATERIALS AND METHODS

Antibodies

Antibodies against modified forms of H3 and H4 histone, HDAC1, and MBD2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against MBD1 and MBD3 were a generous gift from Samson T. Jacob (33). The anti-MeCP2 antibody was kindly provided by Peter L. Jones (9). The anti-DNMT1 (35) and anti-DNMT3b (36) antibodies have been previously described. Anti-DNMT3a antibody was procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture, Reagents, and Treatment with 5-Aza-dC and TSA

The human breast cancer cell lines, MDA-MB-231 and MCF-7, were grown in DMEM supplemented with 5% fetal bovine serum (Gemini Bioproducts, Woodland, CA) and 2 μM L-glutamine (Invitrogen, San Diego, CA). For treatment, cells were seeded at a density of 5 × 10^5/100-mm tissue culture dish. After 24 h of incubation, the culture media were changed to media containing 2.5 μM 5-aza-dC (Sigma Chemical Co., St. Louis, MO) for 96 h or 100 ng/ml TSA (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 12 h. For the combination study, 5-aza-dC was present in culture for 96 h and TSA was added for the last 12 h (4).

Appendix 1

ChIP

ChIP analyses were performed using a published procedure (37) with the following modifications. Chromatin samples were sonicated on ice three times for 10 sec each (*i.e.* until the average length of sheered genomic DNA was 1–1.5 kb) followed by centrifugation for 10 min. The immunoprecipitated DNA was ethanol precipitated and resuspended in 25 μl H_2O. Total input samples were resuspended in 100 μl H_2O and diluted 1:100 before PCR analysis. PCR contained 5 μl of immunoprecipitate or total input, 50 μM of each primer, 1.5 mM $MgCl_2$, 2 mM deoxynucleotide triphosphate mixture, 1× PCR buffer (Sigma), and 1.25 U of *Taq* DNA polymerase (Sigma) in a total volume of 50 μl. The ER promoter was analyzed using the 5′-primer 5′-TGA ACC GTC CGC AGC TCA AGA TC-3′ and the 3′-primer 5′-GTC TGA CCG TAG ACC TGC GCG TTG-3′. Initially, PCR was performed with different numbers of cycles or dilutions of input DNA to determine the linear range of the amplification; all results shown fall within this range. After 30 cycles of amplification, PCR products were run on 1% agarose gel and analyzed by ethidium bromide staining. All ChIP assays were performed at least twice with similar results.

Western Blot

Whole-cell lysates were prepared by scraping cells in 1 ml of ice-cold buffer A [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin]. The lysate was rotated 360° for 1 h at 4 C followed by centrifugation at 12,000 × *g* for 10 min at 4 C to clear the cellular debris (38). Proteins were quantified using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Preparation of nuclear extract was modified from a method described previously (39). Briefly, cells in 10-cm culture dishes were collected for harvesting by gentle scraping in 1 ml ice-cold PBS and pelleting by centrifugation at 1200 rpm at 4 C. The cell pellet (5 × 10^7 cells) was washed once in PBS followed by resuspension in 1 ml of lysis buffer [10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40]. Nuclei were gently isolated by centrifugation at 4,000 rpm and resuspended in 200 μl of extraction buffer [20 mM Tris-Cl (pH 7.9), 0.42 mM KCl, 0.2 mM EDTA, 10% glycerol, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride]. The resulting nuclear extracts were incubated on ice for 10 min and cleared by centrifugation at 10,000 rpm. Protein concentration of nuclear extracts was determined by BCA protein assay kit. Proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes, and Western blot analyses were performed using previously described antibodies. Immunodetection was performed using enhanced chemiluminescence (ECL system, Amersham Pharmacia Biotech, Inc., Arlington Heights, IL) according to the manufacturer's instructions.

RNA Isolation and RT-PCR

Total cellular RNA was extracted using the TRIZOL Reagent kit (Life Technologies, Inc., Gaithersburg, MD) and quantified by UV absorption. RT-PCR was carried out according to our previously described method (3). RNAs under comparison were simultaneously reversibly transcribed to achieve equal efficiency for reverse transcription. Synthesized cDNA (4% or 2 μl, derived from 150 ng of initial RNA) was used for PCR amplification of ER and the constitutively expressed housekeeping gene β-actin. Specific sense and antisense PCR primers used for the amplification across the seventh intron of ER and the first intron of β-actin genes, yielding 470 and 400 bp of PCR products, respectively, were described previously (40). PCR products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Genomic DNA Isolation and Methylation-Specific PCR (MSP) Analysis

Genomic DNA was isolated by standard phenol-chloroform extraction. Isolated DNA was subjected to modification by sodium bisulfite to convert unmethylated cytosines, but not methylated cytosines, to uracils as described previously (41) Methylation status of the bisulfite-modified DNA at the ER locus was characterized by MSP using our previously reported method (32).

Isolation of Histones

Extraction of cellular histones was performed using 2 × 10^6 cells according to a previously published procedure (42) with the following modifications. The acid (H_2SO_4)-soluble supernatant was precipitated with 10 volumes of cold acetone. After overnight precipitation, histones were collected by centrifugation. The pellet containing histones was dissolved in 50 μl of H_2O, and protein was quantified by using the BCA protein assay kit.

Acknowledgments

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